

Malathion resistance and prevalence of the malathion carboxylesterase mechanism in populations of mosquito vectors of disease in Sri Lanka

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Objective To determine the levels of malathion resistance and prevalence of the malathion carboxylesterase mechanism among mosquitoes in Sri Lanka.

Methods Bioassays were carried out using WHO-recommended methods on samples of the following Sri Lankan mosquito vectors: *Culex quinquefasciatus*, *C. tritaeniorhynchus*, *C. gelidus*, *Anopheles culicifacies* B, *A. subpictus*, *Aedes aegypti* and *A. albopictus*.

Findings Malathion-specific carboxylesterase mechanisms were found in *A. culicifacies* and *A. subpictus*, both giving high rates of insecticide metabolism. In contrast, malathion resistance in *C. quinquefasciatus* and *C. tritaeniorhynchus* is linked to broad-spectrum resistance to organophosphorus compounds due to elevated levels of esterases that sequester malaoxon, but are unable to metabolize malathion.

Conclusions Resistance among the *Anopheles* spp. must have occurred as a direct result of antimalarial activities, since malathion use in Sri Lanka is limited to public health treatments. In contrast, resistance among *Culex* spp. has resulted from large-scale use of the organophosphorus insecticide group as larvicides for filariasis control and on rice paddy, where *C. tritaeniorhynchus* predominantly breeds, for agricultural purposes.

Keywords Malathion/metabolism; Carboxylic ester hydrolases/metabolism; Insecticide resistance; Culicidae/metabolism; Culex/metabolism; Anopheles/metabolism; Aedes/metabolism; Prevalence; Sri Lanka (source: MeSH).

Mots clés Malathion/métabolisme; Carboxylic ester hydrolases/métabolisme; Résistance aux insecticides; Moustique/métabolisme; Culex/métabolisme; Anophèles/métabolisme; Aedes/métabolisme; Prévalence; Sri Lanka (source: INSERM).

Palabras clave Malathion/metabolismo; Hidrolasas de ester carboxilico/metabolismo; Resistencia a insecticida; Culicidae/metabolismo; Culex/metabolismo; Anopheles/metabolismo; Aedes/metabolismo; Prevalencia; Sri Lanka (fuente: BIREME).

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Introduction

Mosquitoes play an important role in transmitting diseases such as malaria, dengue, Japanese encephalitis and filariasis. In Sri Lanka, synthetic insecticides have been a major tool used in the country's mosquito control programmes for decades. After the phasing out of use of DDT in the late 1970s, malathion was employed as the main insecticide for malaria control until pyrethroids were introduced to the country between 1995 and 1997.

This extensive use of malathion has contributed to pressure for the selection of resistance to

organophosphorus compounds in some malaria mosquito vector populations (1, 2). The same classes of insecticide that are used for public health purposes are often heavily used in agriculture, and determining the relative importance of these two sources of insecticide resistance selection pressure can be difficult.

Metabolic resistance to organophosphorus compounds in insects is mainly due to quantitative and/or qualitative differences in carboxylesterases (3). Quantitative differences are largely responsible in *Culex quinquefasciatus* and *C. tritaeniorhynchus*; increases in enzyme levels result from gene amplification in both these species (4–6). Qualitative changes in esterases, i.e. mutations causing different esterase alleles in resistant and susceptible insects, can result in the mutated enzyme metabolizing insecticide more rapidly than the wild-type enzyme (7). This phenomenon occurs in malathion-resistant *Anopheles stephensi* in Pakistan and *A. culicifacies* in India, where

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carboxylesterases have high affinity for malathion and turn it over rapidly (7, 8).

In this paper we report the levels of malathion resistance and prevalence of the malathion carboxylesterase mechanism in Sri Lanka, after more than two decades of malathion use against seven mosquito vectors of human disease, and discuss their implications for disease vector control.

Materials and methods

Mosquitoes

Specimens of *C. quinquefasciatus*, the major vector of filariasis, were obtained in Colombo, Western Province, by collecting indoor resting insects. *C. tritaeniorhynchus* and *C. gelidus*, vectors of Japanese encephalitis, were collected from Anuradhapura, North-Central Province, using CDC light traps hung in piggeries. *A. culicifacies* B and *A. subpictus*, vectors of malaria, were collected in cattle-baited traps in Galewela, Central Province. *Aedes aegypti* and *A. albopictus*, vectors of dengue, were collected by human-baited catches in Kandy, Central Province. All collections were undertaken from 1997 to early 1998. The St Mal strain of *A. stephensi* from Pakistan, homozygous for a malathion carboxylesterase resistance mechanism (9), was used as a positive control. Assays were undertaken directly on field-collected mosquitoes after a 24-hour holding period.

Bioassays

Bioassays were carried out to ascertain the level of malathion resistance, if any, in the Sri Lankan mosquito populations. The bioassays were conducted by means of tarsal contact exposure to insecticide-impregnated papers, prepared by standard WHO-recommended methods (10). Solutions of malathion (5% v/v) were prepared by mixing the technical grade insecticide with olive oil. Rectangles of Whatman No.1 filter-paper (12 cm × 18 cm) were impregnated with the insecticide/oil solution at a fixed spreading rate of 3.6 mg/cm². An equal volume of acetone was used to ensure an even distribution of the oil solution on the paper. Papers were left at room temperature until the acetone had evaporated and were then stored, foil-wrapped, at -20 °C until used.

Batches of 18–25 adult mosquitoes were exposed to insecticide-impregnated papers in standard WHO test tubes (12 cm × 4 cm) lined with the papers. All tests were undertaken at 25 °C ± 2 °C and 80% relative humidity. After 1 h, insects were transferred to a clean holding tube, provided with sugar feeders, and held for a 24-h recovery period. For each species a minimum of 100 mosquitoes were tested. Controls were exposed to papers impregnated with olive oil alone.

Monoxygenase content

A crude estimate of monoxygenase content for *A. subpictus*, *A. culicifacies*, and *A. stephensi* was

obtained with individual, field-caught, non-blood-fed females using the assay described by Brogdon et al. (11).

Detection of malathion carboxylesterase activity

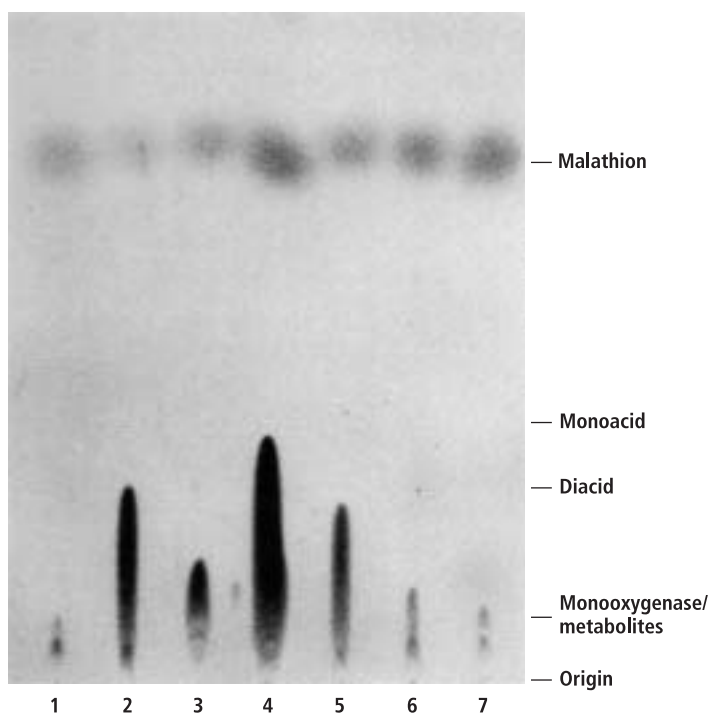
Malathion resistance can result from a number of mechanisms. Resistance caused by a malathion carboxylesterase mechanism results in increased rates of production of the monoacid and/or diacid of malathion in the resistant insects. For each detection experiment, 25–50 adult mosquitoes were homogenized in 1 ml of Tris buffer at a concentration of 25 mmol/l (pH 7.5) and centrifuged at 13 000 *g* for 5 min. The supernatants were adjusted to equal protein concentrations and incubated with malathion at a concentration of 300 µmol/l for 2 h at room temperature. The samples were then extracted with three volumes of chloroform and blown to dryness under a stream of air. The extract was resuspended in 20 µl of chloroform and loaded onto a silica gel thin-layer chromatography (TLC) plate. The plate was eluted with a mobile phase consisting of *n*-hexane: diethyl ether (1:3), then sprayed with a 0.5% (w/v) solution of 2,6-dibromoquinone 4-chlorimide in cyclohexane and left at 100 °C for 2 h to visualize spots of malathion and its metabolites. Distilled water or boiled insect homogenate was incubated with the same concentration of malathion as a negative control. Adult mosquito homogenate (with a protein content equal to that of the field samples) was prepared from the St Mal strain of *A. stephensi* and run as a positive control.

Results

In the bioassays, no mortalities occurred for any insect species after exposure to control papers. A high level of resistance to malathion occurred in *C. quinquefasciatus* (78% survival on the WHO malathion discriminating dosage), *A. culicifacies* (70%) and *C. tritaeniorhynchus* (65%). The level of malathion resistance was lower in *A. subpictus* (15%). Populations of *C. gelidus*, *A. aegypti* and *A. albopictus* were fully susceptible to malathion at this dose.

A carboxylesterase-based resistance mechanism was found in the Sri Lankan anopheline but not the culicine mosquito populations. High levels of mono- and diacid metabolites of malathion were produced by homogenates of *A. culicifacies* and *A. subpictus* incubated with malathion, showing that enzymes in these resistant populations metabolize malathion rapidly (Fig. 1). The rate of malathion metabolism in these two species was faster than that of the highly laboratory-selected malathion-resistant St Mal population of *A. stephensi*, although almost all the malathion was converted to the diacid by St Mal, while both monoacid and diacid metabolites were seen in the field samples. The St Mal *A. stephensi* colony survives 8 h of exposure to 5% malathion in standard WHO bioassays, indicating that the level of

Fig. 1. Malathion carboxylesterase activity determination in field-caught Sri Lankan mosquitoes compared to the St Mal resistant laboratory strain of *Anopheles stephensi* which has a well-defined malathion carboxylesterase resistance mechanism



Lane 1: Boiled insect control
 Lane 2: *Anopheles subpictus*
 Lane 3: *A. stephensi*, St Mal strain
 Lane 4 and 5: *A. culicifacies*
 Lane 6: *Culex tritaeniorhynchus*
 Lane 7: *C. quinquefasciatus*

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resistance to malathion conferred by this mechanism in *A. culicifacies* and *A. subpictus* is likely to be high.

Monooxygenases can contribute to malathion resistance in two ways, by either increasing the rate of metabolism to non-toxic products, or decreasing the rate at which the insecticidal malaoxon is produced from the malathion parent compound. With the mobile phase that we employed for the TLC, monooxygenase metabolites of malathion would be eluted only slightly and would be located at or just above the point where the sample was applied. Fig. 1 shows there is no evidence of any increase in monooxygenase metabolites in any of the species tested.

A decrease in the amount of malaoxon produced would be an unusual but theoretically possible mechanism of malathion resistance. With the TLC system used, malaoxon is eluted to a point equidistant from the monoacid and malathion spots. Over-staining of the TLC plates to reveal malaoxon suggested that similar amounts of this metabolite were produced by all species. The lack of involvement of monooxygenase in the current resistance mechanisms of all the species studied was further supported by haem assays on 30 individual non-

blood fed *A. culicifacies* and *A. subpictus* females. From the haem titrations the maximum estimated equivalent units of cytochrome P-450 were <0.006, which was identical to the *A. stephensi* laboratory colony and suggests no increase in monooxygenase levels. This contrasts with our 1986 data for *A. subpictus* that showed an increase in monooxygenase activity (12).

Discussion

Anopheles and *Culex* mosquito populations in Sri Lanka are under heavy pressure from organophosphorus compounds as adults through indoor house spraying of malathion in malarious areas, and as larvae through the treatment of river beds, water pools, and urban sewage canals with abate and fenthion for malaria and filariasis control, respectively. In addition, *C. tritaeniorhynchus*, which is primarily a paddy-field breeder, is exposed to a range of organophosphorus compounds used in rice cultivation. In Sri Lanka, *C. gelidus*, *A. aegypti* and *A. albopictus* were all susceptible to malathion, probably because of their lower level of exposure than other culicine and anopheline mosquitoes to organophosphorus compounds. In general, these susceptible species do not rest indoors on house walls and they breed in tree holes or other small water bodies, which are unlikely to be sprayed with insecticides.

Sri Lankan *C. quinquefasciatus* and *C. tritaeniorhynchus* populations have resistance mechanisms involving quantitatively changed carboxylesterases, which sequester rather than metabolize malathion (6, 13). These amplified B esterases will bind to malaoxon, the toxic metabolite of malathion, but do not recognize malathion as a substrate. Neither species had a detectable malathion carboxylesterase resistance mechanism, although both mechanisms can coexist, as seen in *C. tarsalis* (14).

When malathion was first introduced in Sri Lanka in 1977, a 20-min exposure to 5% malathion produced 100% mortality in *A. culicifacies* (2). The first *A. culicifacies* survivors at this species-specific discriminating dosage were detected in Sri Lanka in 1979, after two years of malathion spraying. Resistance to the WHO standard *Anopheles* discriminating dosage (exposure to 5% malathion for 1 h) was first observed in 1982, with increased malathion carboxylesterase activity being the major underlying mechanism (2). In contrast, monooxygenases played the major role in malathion resistance in *A. subpictus* in 1987, with no evidence of a malathion carboxylesterase mechanism (1). The oxidase mechanism produced broad-spectrum resistance to organophosphorus compounds, which included a low level of resistance to malathion, and was still the only major mechanism of resistance to these compounds detected in 1991 (12). Although the proportion of *A. subpictus* surviving the WHO discriminating dosage has not risen significantly since 1991, malathion selection pressure, through indoor residual spraying, has now led to its selection of a malathion carboxylesterase resistance mechanism.

Based on our findings on 1997–99 samples, the monooxygenase resistance mechanism appears to have declined from its 1991 levels in *A. subpictus*. This should have resulted in a change in the resistance spectrum in this species, the malathion carboxylesterase conferring a much narrower spectrum than the monooxygenases.

In this study, the levels of malathion metabolic products produced in both *A. culicifacies* and *A. subpictus* were higher than those from a homozygous resistant laboratory colony of *A. stephensi* that is ca 20-fold more resistant to malathion, suggesting that the malathion carboxylesterases are efficient resistance mechanisms in both these *Anopheles* species in Sri Lanka. Since malathion has not been used for anything other than malaria control in Sri Lanka since the early 1980s, this resistance must have been selected as a direct result of antimalarial activities. The malathion carboxylesterase mechanism has been selected in addition to the earlier monooxygenase resistance mechanism in *A. subpictus*, presumably because the latter mechanism did not provide complete protection against the level of malathion used for malaria control operations.

A. culicifacies continues to exhibit narrow spectrum malathion resistance resulting from the carboxylesterase mechanism, correlated with the high rates of malathion coverage in Sri Lanka. The frequency of resistant individuals, as judged from WHO bioassays, has risen since the 1980s. The high levels of malathion metabolism in the two Sri Lankan *Anopheles* spp. compared to the laboratory homozygous malathion-resistant *A. stephensi* are unusual. The metabolic rates seen in the Sri Lankan mosquitoes in the present study will probably underestimate the maximal rate if they follow the patterns observed in *A. stephensi*, where malathion carboxylesterase activity peaks in one-day-old adult insects and then declines as the adults age (15). ■

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Conflicts of interest: none declared.

Résumé

Résistance au malathion et prévalence du mécanisme de la malathion carboxylestérase dans des populations de moustiques vecteurs de maladies à Sri Lanka

Objectif Déterminer les taux de résistance au malathion et la prévalence du mécanisme de la malathion carboxylestérase chez les moustiques à Sri Lanka.

Méthodes Des tests biologiques ont été effectués selon les méthodes recommandées par l'OMS sur des échantillons des moustiques vecteurs suivants présents à Sri Lanka : *Culex quinquefasciatus*, *C. tritaeniorhynchus*, *C. gelidus*, *Anopheles culicifacies* B, *A. subpictus*, *Aedes aegypti* et *A. albopictus*.

Résultats Des mécanismes faisant intervenir une carboxylestérase spécifique du malathion ont été trouvés chez *A. culicifacies* et *A. albopictus*, espèces qui métabolisent fortement les insecticides. En revanche, la résistance au malathion chez *C. quinquefasciatus* et

C. tritaeniorhynchus est liée à une résistance à large spectre aux organophosphorés due à un taux élevé d'estérases qui séquestrent le malaaxon tout en étant incapables de métaboliser le malathion.

Conclusion La résistance chez *Anopheles* spp. est probablement le résultat direct des activités de lutte antipaludique, car l'utilisation du malathion à Sri Lanka se limite aux traitements effectués aux fins de santé publique. En revanche, la résistance chez *Culex* spp. résulte de l'utilisation à grande échelle des insecticides du groupe des organophosphorés en tant que larvicides pour la lutte contre la filariose, et pour le traitement agricole des rizières, principaux gîtes larvaires de *C. tritaeniorhynchus*.

Resumen

Resistencia al malatión y prevalencia del mecanismo de la malatión-carboxilestérasa en las poblaciones de mosquitos vectores de enfermedades en Sri Lanka

Objetivo Determinar los niveles de resistencia al malatión y la prevalencia del mecanismo de actividad carboxilestérasa sobre este producto entre los mosquitos en Sri Lanka.

Métodos Empleando métodos recomendados por la OMS, se llevaron a cabo bioensayos en muestras de los siguientes mosquitos vectores de Sri Lanka: *Culex quinquefasciatus*, *C. tritaeniorhynchus*, *C. gelidus*; *Anopheles culicifacies* B, *A. subpictus*; *Aedes aegypti* y *A. albopictus*.

Resultados Se detectaron mecanismos de actividad carboxilestérasa específicos para el malatión en *A. culicifacies* y *A. subpictus*, dato indicativo de una alta tasa de metabolización del insecticida. Por el contrario, la resistencia de *C. quinquefasciatus* y *C. tritaeniorhynchus* al malatión está mediada por una resistencia de amplio espectro a los compuestos organofosforados, debida a unos niveles elevados de esterases que secuestran el malaaxon pero son incapaces de metabolizar el malatión.

Conclusión La resistencia desarrollada por *Anopheles* spp. tiene que ser una consecuencia directa de las actividades de lucha antipalúdica, dado que en Sri Lanka el malatión sólo se emplea con fines de salud pública. La resistencia observada en *Culex* spp., en cambio, se debe

al uso en gran escala de insecticidas organofosforados como larvicidas contra la filariasis, y con fines agrícolas en arrozales, espacios preferidos como criaderos por *C. tritaeniorhynchus*.

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